Variation Among Geographically Separated Populations of *Dermacentor andersoni* (Acari: Ixodidae) in Midgut Susceptibility to *Anaplasma marginale* (Rickettsiales: Anaplasmataceae)

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ABSTRACT *Anaplasma marginale* is a tick-borne rickettsial pathogen of cattle that is endemic throughout large areas of the United States. Cattle that survive acute infection become life-long persistently infected carriers. In the intermountain west the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles, is the most common vector of *A. marginale*. Male *D. andersoni* acquire *A. marginale* when feeding on persistently infected cattle and biologically transmit it when they transfer from infected to susceptible hosts. Host-seeking adult *D. andersoni* were collected from four widely separated natural populations and tested for susceptibility to midgut colonization with *A. marginale*. Male ticks were fed on calves persistently infected with a strain of *A. marginale* naturally transmitted by *D. andersoni*. Gut infection rates ranged from 12.5% of ticks collected from a mountain site near Hamilton, MT, to 62.5% of ticks from a rangeland site near Riley, OR. Sites near Miles City, MT, and Kamloops, British Columbia, Canada, had intermediate levels of susceptibility. The infection rates differed significantly among populations, and the same populations sampled in two consecutive years were not significantly different from one year to the next. Although there was variation among the populations in the size of ticks, size was unrelated to acquisition of gut infection. Quantitative polymerase chain reaction (PCR) demonstrated that there was no significant difference between populations in the mean number of genome copies in the guts of infected ticks. *A. marginale* from infected ticks was genotyped to confirm that they were all infected with the laboratory strain, and a sample of 682 field-collected *D. andersoni* was surveyed for *A. marginale* by nested PCR; none were found to be naturally infected. Infection of the gut is an essential constituent of vector competence for *A. marginale*; in this study, we have demonstrated significant variation among populations in this key component of vector competence.

KEY WORDS *Dermacentor andersoni*, *Anaplasma marginale*, vector competence, quantitative PCR, survey

Anaplasma marginale is a rickettsial pathogen of cattle that is biologically transmitted by ticks in the western United States and also can be mechanically transmitted by biting flies and fomites (Kocan et al. 2003). After transmission to a susceptible host *A. marginale* invades erythrocytes; in the acute phase of infection rickettsemia may exceed 10^9 infected erythrocytes (IE) per milliliter of blood and can result in clinical anaplasmosis, which is characterized by anemia, weight loss, abortion, and usually death. Surviving animals develop a life-long persistent infection characterized by repeated cycles of rickettsemia ranging from 10^2 to 10^7 IE/ml, below the threshold for microscopic detection on stained blood smears (Kieser et al. 1990, French et al. 1998). Persistently infected cattle show no clinical signs of infection and serve as reservoirs of the pathogen.

The Rocky Mountain Wood tick, *Dermacentor andersoni* Stiles, is the predominant biological vector of *A. marginale* in the northwestern United States. Although transstadial transmission of *A. marginale* by *D. andersoni* has been demonstrated (Stitch et al. 1989), immature stages of this species feed on small mammals that are not susceptible to *A. marginale*. Because *A. marginale* is not transovarially transmitted (Stitch et al. 1989), the currently accepted model for transmission of *A. marginale* by *D. andersoni* is via interhost transfer of adult male ticks (Zaugg et al. 1986). In the early spring, host-seeking adult ticks acquire the pathogen by feeding on persistently infected cattle, the midgut epithelium is the initial site of infection. The ability of *A. marginale* strains to infect this tissue has been proposed to be a determinant of tick transmissibility (de la Fuente et al. 2001). After replication in the gut, *A. marginale* disseminates and

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infects other tick tissues, including the salivary glands. Replication in the salivary glands seems to be linked to transmission feeding. *A. marginale* is transmitted to the subsequent host by means of the saliva. The feeding and mating habits of male *D. andesoni* facilitate transmission. An initial few days of blood feeding is required for sperm maturation in *D. andersoni*, after this males move about on the host in response to pheromone produced by feeding females (Homsher and Sonenshine 1976). During this time, males feed intermittently. Contact between cattle results in movement of ticks from one host to another, and transmission occurs when infected males transfer to and feed on susceptible individuals in the population (Stiller et al. 1989a). Once male ticks become infected they remain infected for life and can transmit the pathogen at each subsequent feeding; serial transmission by male ticks to six consecutive cattle has been demonstrated experimentally (Kocan et al. 1992).

Both *A. marginale* infection prevalence and disease incidence vary markedly within the region where *D. andersoni* is the predominant vector (Saulmon 1962, Long et al. 1974, Torioni de Echaide et al. 1998, Van Donkersgoed et al. 2004); factors responsible for this variation have not been investigated. The existence of "nontick-transmissible" isolates of *A. marginale* (Smith et al. 1986, Wickwire et al. 1987) and quantitative differences between *A. marginale* strains within the same tick vector species (Futse et al. 2003) indicate that pathogen strain can affect the efficiency of transmission. The reverse, the effect of tick strain on *A. marginale* transmission, has not been investigated. *D. andersoni* is widely distributed across a range of habitats from western Nebraska and the Dakotas west to the Cascade and Sierra Nevada Mountains and from northern New Mexico and Arizona to southern British Columbia and Alberta (the "intermountain west"). Although genetic variation among populations of this species has not been studied, there is some evidence of genetic differences between populations. Populations from British Columbia cause tick paralysis on cattle at a much higher rate than do populations from Alberta (Wilkinson 1985); this ability to cause tick paralysis can be increased by selection, consistent with a genetic basis (Lysyk and Majak 2003). Populations collected from "prairie" and "montane" regions within Alberta differ in their attachment site preferences on cattle (Wilkinson 1972, 1985) and in their responses to photoperiod (Pound and George 1991). These interpopulation differences in the vector together with variation in the prevalence of *A. marginale* in cattle suggests that there may be variation among tick populations in vector competence for *A. marginale.* The population genetics of this species has not been studied, nor have there been any studies comparing vector competence of *D. andersoni* from different geographic locations for *A. marginale* or any other pathogen.

We hypothesize that *D. andersoni* populations from different geographic locations differ in their ability to acquire and be colonized by *A. marginale.* To test this hypothesis, we collected *D. andersoni* from four widely separated regions and quantified the percentage of ticks that acquired midgut infection after feeding on calves that were persistently infected with the St. Maries strain of *A. marginale.* Acquisition feeding during the persistent phase of the infection replicates the most likely field situation, and use of a single *A. marginale* strain eliminates differences that could be attributed to the pathogen itself, allowing for testing of variation among tick populations.

Materials and Methods

Tick Collection. Host-seeking adult *D. andersoni* ticks were collected from 18 sites in the northwestern United States and southwestern Canada by flagging and/or dragging in the spring of 2002 and 2003; the exact coordinates (latitude and longitude) and elevation of each collection site are presented in Table 1. Adult male ticks from four of these sites were tested for susceptibility to midgut infection with *A. marginale.* These sites were 1) Lake Como, MT, along a hiking trail on the south facing slope to the north of Lake Como, 20 km south of Hamilton, MT; this is a woodland site with the ticks collected primarily from grassy areas along the edge of the trail. 2) Miles City, MT, on the Fort Keogh Agricultural Experiment Station, directly southwest of Miles City; this is an open range site with primarily grasses and isolated sagebrush. 3) Walker Lake, Alberta, on a south facing slope to the north of Walker Lake, 15 km south of Kamloops, British Columbia; vegetation at this site is primarily bunch grasses and sagebrush. 4) Placidea Butte, OR, on the Northern Great Basin Experimental Range, 16 km west of Riley, OR; the vegetation is primarily sagebrush and bunch grasses, ticks were collected primarily from south facing slopes with isolated trees and rocky outcroppings. With the exception of Lake Como, which is a forest site with no nearby cattle grazing, these sites were all active cattle ranges. Placidea Butte is in an area where the *Anaplasma* prevalence has historically been high (Peterson et al. 1977); Miles City and Lake Como are areas where recent data suggests there is a low prevalence of *Anaplasma* (Van Donkersgoed et al. 2004); no information is available regarding the prevalence of infection at the Walker Lake site. Additional ticks were collected from three of these four sites, as well as the 14 others to be tested for the prevalence of *A. marginale* in field-collected adult ticks (Table 1). All ticks were returned to the laboratory, sexed, counted, and held in an incubator at 15°C, 98.5% RH (over saturated K_2SO_4; Winston and Bates 1960). Ticks not needed for the *Anaplasma* acquisition studies were preserved in 70% ethanol until DNA isolation.

A few days before being acquisition fed on infected hosts, ticks from each population were uniquely marked by removing one or a combination of the second, third, or fourth legs so that all ticks could be fed together at the same time under the same feeding patch and still be distinguished by their population of origin. Legs were removed with a hot knife to cauterize the wound, preventing hemolymph from bleed-

Table 1. *D. andersoni* ticks collected in spring 2002 and 2003 tested for acquisition of midgut infection, and ticks collected in 2002 tested by nested PCR to determine prevalence of infection with *A. marginal*

Location	State or province	Coordinates[a]		Elevation (m)	Acquisition of Midgut infection[b]		PCR[c]	
		Latitude (N)	Longitude (W)		2002	2003	Male	Female
Douglas Lake	BC	50° 11′	120° 25′	850			10	10
Kamloops	BC	50° 43′	120° 26′	380			10	27
Shumway Lake	BC	50° 31′	120° 16′	900			17	51
Walker Lake	BC	50° 33′	120° 15′	700	24	24		
Chin Lake	Alberta	49° 36′	112° 11′	910			20	18
Onefour	Alberta	49° 07′	110° 28′	920			16	16
Finch Farm	WA	46° 41′	117° 27′	500			14	22
Hayes Farm	WA	46° 42′	117° 23′	600			22	19
Placidea Butte	OR	43° 28′	119° 45′	1,400	24	33	13	9
Umatilla	OR	45° 05′	118° 59′	920			17	13
Colgate Licks	ID	46° 28′	114° 56′	920			29	26
Dubois	ID	44° 14′	112° 10′	1,670			13	19
Moscow Mt.	ID	46° 47′	116° 52′	1,020			22	16
Lake Como	MT	46° 04′	114° 15′	1,320	24	24	19	55
Miles City	MT	46° 19′	105° 59′	760	24	24	23	50
Skalkaho	MT	46° 11′	113° 54′	1,560			10	10
North Platte	WY	41° 03′	106° 23′	2,470			10	20
Curt Gowdy S.P.	WY	41° 16′	105° 19′	2,380			19	17
Total							284	398

[a] Exact latitude and longitude of tick collection sites.
[b] Number of ticks field collected in 2002 and 2003 and used for studies on acquisition of midgut infection.
[c] Number of ticks field collected in 2002 and tested by nested PCR for *A. marginale.*

ing out through the cut leg. The first pair of legs was left uncut to avoid damaging the Haller's organ.

Tick Feeding. Acquisition feedings were done on Holstein calves that had reached the persistent phase of infection with the St. Maries strain of *A. marginale.* St. Maries is a tick-transmissible strain originally isolated from *D. andersoni* ticks collected off of an acutely infected bull in northern Idaho (Eriks et al. 1994). Cattle were PCR positive for *A. marginale* at the time of the acquisition feedings and blood samples were collected for determination of rickettsemia by quantitative real-time PCR, as described below. Cattle used in these studies were cared for following protocols approved by the University of Idaho Institutional Animal Care and Use Committee.

Male ticks collected in 2002 were acquisition fed on calf #c931bl 225 d postinfection (p.i.) with the St. Maries strain. Male ticks collected from the same sites in 2003 were fed on calf #c995bl 70 d p.i. In both strains, the infection was undetectable in blood smears, consistent with a level of $<10^7$ IE/ml, throughout the acquisition feeding interval (23 July–25 July 2002 and 20 July–22 July 2003, respectively), the actual levels of infection were determined by real-time PCR. Within each trial all ticks were fed at the same time under the same feeding patch. Feeding patches were made of stockinet and muslin and were glued to the side of the calf with hip tag cement (Bug Bull Hip Tag Cement, Bigley Supply Co., Elysian, MN). Ticks were placed on the calf at 8:00 a.m. on day 0 and all unattached ticks removed 10 h later; at 6:00 p.m. on day 2 only the attached ticks were collected, so that all ticks that were used for the experiment had been attached from 48 to 58 h.

Fed ticks were sorted into their populations of origin based on their leg cut markings and were individually weighed within 24 h of being removed from the host. Acquisition fed ticks were held at 23.0 ± 1.5°C over saturated K_2SO_4 (98.5% RH) as described for 8–10 d postremoval before dissection. Ticks were dissected in Hanks' balanced salt solution (Sigma, St. Louis, MO). Each tick was dissected on a fresh piece of dental wax with a new razor blade and clean forceps. Forceps were thoroughly cleaned between ticks by sonication in 5% SDS for 5 min, followed by two rinses with sterile distilled H_2O and then dipping and flaming in 95% ethanol. Midguts and salivary glands were dissected from each tick and placed in 100 μl of proteinase K buffer with 2× enzyme (0.01 M Tris, pH 7.8, 0.005 M EDTA, 0.5% SDS, and 100 μg/ml proteinase K) and frozen at −20°C until DNA preparations were made.

DNA Preparation and PCR. Dissected guts and salivary glands were thawed and incubated in the proteinase K solution at 50°C for 1 h. After incubation, 100 μl of IsoQuick lysis solution was added (Orca Research, Bothel, WA), and the tissue was triturated with a disposable plastic pestle (Bel-Art Products, Pequannock, NJ). After grinding, tubes were incubated for an additional hour at 50°C. DNA was extracted from the lysate by using the IsoQuick DNA extraction kit (Orca Research) following the modified protocol of Schwartz et al. (1997) and with 1.0 μl of a 20 mg/ml solution of glycogen (Roche Diagnostics, Indianapolis, IN) added before the DNA precipitation step to improve the yield of small quantities of DNA. DNA was resuspended in 30 or 50 μl of H_2O and stored in the refrigerator until use.

Tick tissues were tested for *A. marginale* by using a nested PCR test that was modified from Torioni de Echaide et al. (1998) by the design of an additional primer to make the hemi-nested PCR described by the authors fully nested. The primer sequences are based on the *A. marginale msp5* sequence in GenBank (accession no. M93392). The external primer pair MSP-5 254 F: 5′-GCA TAG CCT CCG CGT CTT TC-3′ and the new primer MSP-5 779R: 5′-ACA CGA AAC TGT ACC ACT GCC-3′ amplify a predicted fragment of 525 bp by using an annealing temperature of 65°C. The internal (nested) primer pair MSP-5 367 F: 5′-TAC ACG TGC CCT ACC GAG TTA-3′ and MSP-5 710R: 5′-TCC TCG CCT TGG CCC TCA GA-3′ amplify a predicted fragment of 343 bp by using an annealing temperature of 55°C. *A. marginale* genomic DNA was run as a positive control. One of every eight reactions was a negative control. For the negative controls, water was substituted for template in the first round reaction, and the product of the first round negative control served as the template for the second round negative control.

As a control for the quality of the DNA preparations, all samples that were negative for *A. marginale* were tested for the presence of tick DNA by using primers 16s+1: 5′-CCG GTC TGA ACT CAG ATC AAG T-3′ and 16s-1: 5′-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3′ for a 450-bp fragment of the tick mitochondrial 16s rDNA (Norris et al. 1996).

Confirmation of *A. marginale* Strain. Because these studies were conducted with field-collected ticks, there is a possibility that some of them might have been naturally infected with *A. marginale* before collection. The number and sequence of repeats in the *msp1α* gene provide a stable strain marker that allows definitive identification of *A. marginale* strains (Allred et al. 1990, Palmer et al. 2001, de la Fuente et al. 2002). PCR amplification and sequencing of *msp1α* was conducted on a sample of the infected ticks to confirm that they were infected with the St. Maries strain of *A. marginale* used in the experiment. The methods followed those of Palmer et al. (2001) with some modifications. The forward primer was as described, MSP1a.For: 5′-ATT TCC ATA TAC TGT GCA G-3′. The reverse primer described in Palmer et al. (2001) is located at a site that is not conserved for St. Maries; a new primer was chosen 27 bases downstream at a more conserved location, *msp1α*.Rev: 5′-ATG TAC TCA ACA CTC GC-3′. These primers were optimized at an annealing temperature of 58°C and amplify a fragment of 564 bp from the St. Maries strain. Amplification of *msp1α* was attempted from all of the ticks that were nested PCR positive for *msp5.* An aliquot of the amplicon was run on a gel to confirm the size of the fragment and then cloned and sequenced from one or more infected ticks from each population to confirm the strain identification. For cloning, a sample of the *msp1α* PCR products were gel purified (QIAGEN) and TA cloned into the pCR4-TOPO vector with the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). Clones were sequenced by a commercial lab (Amplicon Express, Pullman, WA) by using the T3 priming site on the cloning vector. The sequences were assembled using the SeqMan II module of the LaserGene sequence analysis package (DNAstar, Madison, WI) and the consensus sequences aligned with the MegAlign module of LaserGene. The sequences from the ticks were compared with the sequence from the blood of the infected cattle and with the St. Maries *msp1α* sequence in GenBank (accession no. AF293062).

Testing Field-Collected *D. andersoni* for *A. marginale.* As a further demonstration that field-collected questing ticks would not be expected to be naturally infected with *A. marginale*, 682 adult ticks (284 males and 398 females) collected from 17 sites throughout the intermountain west in spring 2002 were tested for infection with *A. marginale.* Collection locations and numbers and sexes of ticks tested from each site can be found in Table 1. Genomic DNA was prepared from whole ticks as described previously (Scoles 2004) and tested with the nested *msp5* PCR described above. Five ticks from the Lake Como population fed on a St. Maries strain infected calf (12.5% infection rate) and 12 from the Miles City population identically fed (33.3% infection rate) were included as blind, randomly assorted positive controls. In addition, one *A. marginale* genomic DNA-positive control was run for each of 41 ticks, and one negative control (by using water instead of template) was included for each of six to seven ticks as described previously.

Confirmation of Tick Species. Field-collected *D. andersoni* ticks were identified to species based on morphological characters using the key of Yunker et al. (1986). The species identification was confirmed by sequencing a 450-base fragment of the mitochondrial 16s rDNA from four to six ticks from each population in each year of the study. Ticks that were sequenced included both infected and uninfected individuals. The fragment was PCR amplified using the primers 16s+1 and 16s-1, as described previously (Norris et al. 1996). The amplicons were TA cloned as described, and clones were sequenced by a commercial laboratory (Amplicon Express) with a single sequencing reaction by using the T3 or T7 priming sites on the vector. The sequences for two to five clones from each tick were assembled with LaserGene as described previously. Sequences from field-collected ticks were compared with *D. andersoni* sequences from GenBank (accession nos. L34299, AF309032, and AF309031) for confirmation of identifications.

Quantitative PCR. Quantitative real-time PCR was carried out on infected blood collected at the time of tick acquisition feeding and on all of the PCR-positive guts and salivary glands from the ticks collected in 2003. The TaqMan quantitative PCR protocol described by Futse et al. (2003) was used with the exception that the standard curve was established using the 525-base *msp5* fragment amplified by the primers described above and TA cloned into the pCR4-TOPO vector as described previously. Only samples that were first shown to be positive for *A. marginale* by nested PCR were tested with quantitative PCR. Samples of 5 μl of the extracted DNA from the blood of

Table 2. Gut infection rates of male *D. andersoni* collected in spring 2002 and 2003 from four different populations and fed on calves persistently infected with *A. marginale*

Collection site (pop)	No. positive/total (%)	
	2002[a]	2003[b]
Lake Como, MT	3/24 (12.5)	8/24 (33.3)
Miles City, MT	8/24 (33.3)	8/24 (33.3)
Walker Lake, AB	12/24 (50.0)	12/24 (50.0)
Placidea Butte, OR	15/24 (62.5)	18/33 (54.5)

Differences between years for each population were not statistically significant.

[a] Rickettsemia of calf, 2.94×10^5 organisms/ml.

[b] Rickettsemia of calf, 4.75×10^6 organisms/ml.

each infected calf and from each positive tick gut were tested in triplicate; results were expressed as a mean number of *A. marginale* genome copies per ml of blood, or per tick gut.

Statistical Analysis. Numbers of infected and uninfected individuals within each population were compared using the *G* test of goodness-of-fit for single-classification frequency distributions and applying Williams' correction for sample sizes <200 (Sokal and Rohlf 1987). Weights of infected and uninfected ticks within each population were compared using Student's *t*-test. Quantitative PCR data were compared between populations by using Student's *t*-test.

Results

Variation in *A. marginale* Infection between Populations. Previous studies have shown that *A. marginale* DNA from the Florida strain, a nontick-transmissible strain that does not colonize the *D. andersoni* midgut, is not detectable in the gut of exposed ticks beyond the second day postremoval (Stiller et al. 1989b). Therefore, tick midguts that were positive 8–10 d postremoval represent pathogen invasion and colonization of the midgut. The ticks collected in spring 2002 were fed on a persistently infected calf with a rickettsemia of 2.94×10^5 (5.468 $\log_{10}$) organisms per milliliter of blood. Midgut infection rates in these ticks ranged from a low of 12.5% (3/24) for the Lake Como population to a high of 62.5% (15/24) for the Placidea Butte population. The percentage of individuals that acquired midgut infections with *A. marginale* was dependent on population ($G_{adj} = 14.65$, $P < 0.005$) (Table 2).

The ticks collected in spring 2003 were fed on a persistently infected calf with a rickettsemia of 4.75×10^6 (6.677 $\log_{10}$) organisms per milliliter of blood. The infection rates for the 2003 ticks were, on average, higher than for the 2002 collections, consistent with the >16-fold higher rickettsemia of the acquisition host. Midgut infection rates ranged from a low of 33.3% (8/24) for both the Lake Como and the Miles City populations, to a high of 54.5% (18/33) for the Placidea Butte population. Although the trend was the same as for the 2002 populations (i.e., the most susceptible and the least susceptible populations were the same for the two years), and two of the populations had the same level of infection in the two different years, there was not a statistically significant relationship between population and rate of infection for the ticks collected in 2003 ($G_{adj} = 3.95$, $P > 0.5$) (Table 2).

Two populations, Miles City and Walker Lake, had the same gut infection rate in 2002 and 2003, 33.3 and 50%, respectively. In both years, the populations with the highest (Placidea Butte) and the lowest (Lake Como) infection rates were the same (Table 2), and the year-to-year differences between the two populations were not significant (Placidea Butte 2002 versus 2003, $G_{adj} = 0.35$, $P > 0.9$; Lake Como, $G_{adj} = 2.90$, $P > 0.1$).

Over the 2 yr of the study, only four ticks had salivary gland infections. In 2002, two ticks from the Placidea Butte population were salivary gland positive. In 2003, one tick from Placidea Butte and one tick from Walker Lake were salivary gland positive.

Tick Size (Weight) Has No Effect on Infection. Although there was considerable variation in the size (weight) of ticks, both within and between populations, there was no significant difference between the mean weights of infected and uninfected ticks in any of the populations in either 2002 or 2003 (Table 3). The greatest size variation occurred in the Lake Como population in 2002; these ticks ranged in size from 3.64 to 21.14 mg (median 6.31 mg.). The size of ticks, on average, from the Lake Como, Miles City, and Walker Lake populations were not significantly different from one another, whereas ticks from the Placidea Butte population were, on average, significantly smaller than ticks from the other three populations in both years of the study (*t*-test: 2002, $P = 5.1 \times 10^{-8}$; 2003, $P = 8.6 \times 10^{-12}$).

Confirmation of *A. marginale* Strain. The *msp1α* amplicons from experimentally infected ticks and

Table 3. Relationship between mean weights of *A. marginale*-infected and-uninfected *D. andersoni*

Collection site	Yr of collection	Avg wt		*t*-test *P* value
		Infected ± SEM (*n*)	Uninfected ± SEM (*n*)	
Lake Como, MT	2002	10.84 ± 3.22 (3)	7.36 ± 0.94 (21)	0.21763
	2003	6.60 ± 0.64 (8)	7.51 ± 0.81 (16)	0.46598
Miles City, MT	2002	7.93 ± 0.49 (8)	6.95 ± 0.43 (16)	0.17443
	2003	5.90 ± 0.48 (8)	6.54 ± 0.62 (16)	0.50943
Walker Lake, AB	2002	6.65 ± 0.50 (12)	6.94 ± 0.50 (12)	0.16536
	2003	7.33 ± 0.49 (12)	6.38 ± 0.53 (12)	0.20356
Placidea Butte, OR	2002	3.94 ± 0.21 (15)	3.69 ± 0.26 (9)	0.45660
	2003	3.71 ± 0.20 (18)	3.63 ± 0.22 (15)	0.79529

from the blood of the persistently infected calves were 564 bp, the length expected for the St. Maries strain of *A. marginale.* The *msp1α* fragment was sequenced from at least one tick from each population and from the blood of the infected calves. All fragments had >99% identity with each other and with the *A. marginale* St. Maries strain *msp1α* sequence in GenBank (accession no. AY010245), indicating that all the *A. marginale*-positive ticks were infected with the St. Maries strain to which they were experimentally exposed.

No Infection of Field-Collected *D. andersoni*. None of the 284 male or 398 female field-collected *D. andersoni* that were tested for *A. marginale* were positive. Three of 17 field-collected ticks that were acquisition fed in the laboratory on an *A. marginale*-infected calf and included with the unexposed field collected ticks as blind, randomly assorted positive controls were positive. Of the five laboratory-exposed ticks from the Lake Como population, a single tick was positive and of the 12 laboratory-exposed ticks from the Miles City population, two were positive.

Confirmation of Tick Species Identification. All of the ticks tested from each population were confirmed to be *D. andersoni* based on mitochondrial 16S rDNA sequence identity with known *D. andersoni* sequences in GenBank.

Quantitative PCR. Quantitative PCR for *A. marginale* in tick midgut tissues was carried out only on ticks collected in 2003. Only tick samples that were positive for *A. marginale* by nested PCR were tested with quantitative PCR; because nested PCR is more sensitive than quantitative PCR, not all of the ticks that were nested PCR positive had quantifiable amounts of *A. marginale.* For the ticks with quantifiable amounts of *A. marginale* in their guts, there was no significant difference between populations in the mean number of genome copies of *A. marginale* in the gut (Fig. 1A). Amounts of *A. marginale* in the guts of individual ticks ranged from 2.18×10^2 ($2.34 \log_{10}$) organisms in a tick from the Walker Lake population to 2.83×10^4 ($4.45 \log_{10}$) organisms in a tick from Placidea Butte; the median was 1.75×10^3 ($3.24 \log_{10}$) and the overall mean was 3.85×10^3 ($3.59 \log_{10}$). When the number of copies of *A. marginale* was adjusted for tick weight, the differences between populations were still not significant (Fig. 1B). None of the salivary glands that were positive by nested PCR had quantifiable amounts of *A. marginale.*

Discussion

Intraspecific variation in vector competence has been demonstrated for a variety of vector-borne

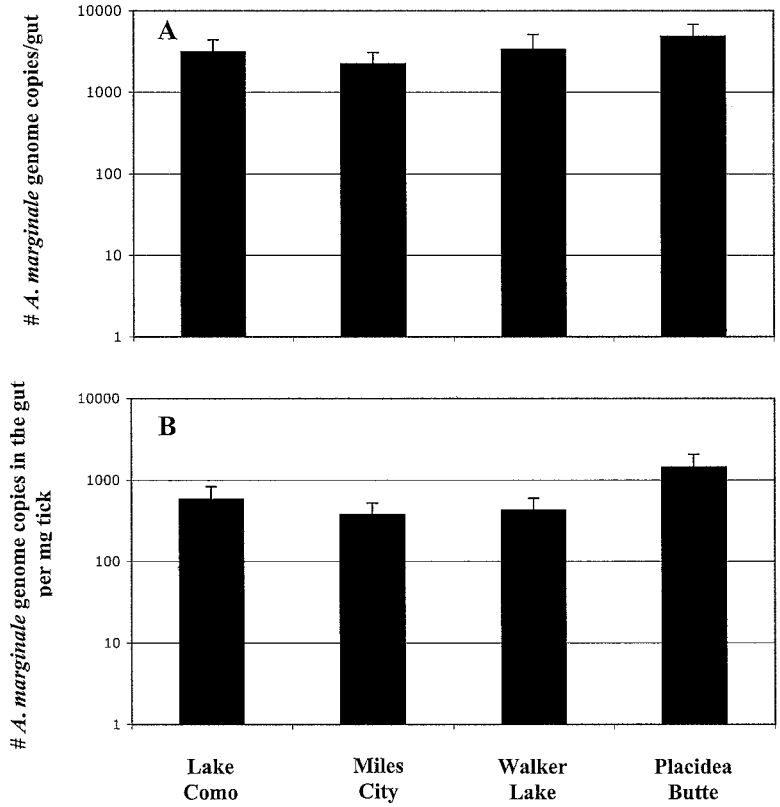

Fig. 1. Amount of *A. marginale* in tick guts based on quantitative (real-time) PCR. (A) *A. marginale* genome copies per tick gut. (B) Adjusted for tick weight; genome copies per tick gut per mg tick (error bars ±1 standard error of the mean).

pathogens, and many of these studies have shown that there is a genetic basis for this variation (James and Fallon 1996, Woodring et al. 1996). Whether genetics accounts for or contributes to the variation in pathogen acquisition seen in the current study is unknown. There is some evidence for genetic differences among populations of *D. andersoni.* Most recently, it has been shown that populations of this species differ in their ability to cause tick paralysis; these differences have a genetic basis because they can be enhanced by selection (Lysyk and Majak 2003). Differences among populations in attachment site preferences on cattle and in their responses to photoperiod also have been described (Wilkinson 1972, 1985; Pound and George 1991), although it is not known whether there is a genetic basis to this variation. Populations of *D. andersoni* collected from the east side and the west side of the Bitterroot valley in western Montana have been shown to differ markedly in infection prevalence with nonpathogenic and pathogenic *Rickettsia,* the rickettsial symbiont *Rickettsia peacockii* and the etiologic agent of Rocky Mountain spotted fever, *Rickettsia rickettsii,* respectively (Niebylski et al. 1997). However, the most likely cause of these differences is the interaction between the symbiont and the pathogen and may not have anything to do with genetic variation in tick susceptibility to one or the other. Whether the variation in susceptibility of *D. andersoni* to *A. marginale* that we have observed in this study is the result of genetics, symbiont–pathogen interactions, environmental factors, some other unidentified factor, or combination of factors is not known; however, the current study demonstrates that intraspecies variation in susceptibility does occur.

D. andersoni fed experimentally for 6–7 d on acutely infected animals with rickettsemia $\geq 10^9$ IE/ml have infection rates approaching 100% (Eriks et al. 1994). However, because acute infection is transient, ticks in natural settings are much more likely to encounter persistently infected hosts with much lower rickettsemias of $\leq 10^7$/ml. Few studies have examined infection rates of ticks fed on cattle with low rickettsemias. In one study, the infection rate of *D. andersoni* fed for 6 d increased with higher levels of rickettsemia, ranging from an infection rate of 27% for ticks fed on a calf with a rickettsemia of 4.0 $\log_{10}$/ml, to 81% for ticks fed at 5.8 $\log_{10}$/ml (Eriks et al. 1993). In another study, *D. andersoni* that were fed for 7 d on cattle with rickettsemias ranging from 5.78 to 6.23 $\log_{10}$/ml had infection rates of 84–90% (Futse et al. 2003). In a similar way, male tick feeding times of shorter duration also may more accurately represent what is likely to occur in a natural setting. After an initial longer duration feed during which sperm maturation occurs, male *D. andersoni* are much more likely to take intermittent blood meals of shorter duration as they move around on hosts in search of females to mate. As a result, the low level rickettsemia and short feeding time used in this study should be much more representative of what ticks actually encounter in natural settings. This feeding method should give the ticks an exposure closer to the threshold level for gut infection, ensuring that only those ticks most competent for initial *Anaplasma* infection and replication will become infected

Although nontick-transmissible isolates of *A. marginale* have been collected from the midwestern and the southeastern United States, none have been collected from the intermountain west where *D. andersoni* is the predominant vector of *A. marginale.* In this study, we used a single strain of *A. marginale* to eliminate strain differences as a variable. All of the infected ticks examined after feeding on an *A. marginale*-infected calf contained the same strain as determined by the strain-specific PCR. As part of this study, we also surveyed a large sample of field-collected *D. andersoni* for *A. marginale,* no natural infections of field-collected ticks, male or female, were identified. This provides additional evidence that the field-collected ticks used in this study are unlikely to have been naturally infected. This survey data also provides some of the only empirical evidence from field-collected ticks in support of the intrastadial transmission model (adult male acquisition and transmission) for *A. marginale;* if interstadial transmission (nymphal acquisition, adult transmission) were important in the epidemiology of *A. marginale* we would have expected to find some naturally infected adults, both male and female, that acquired infection during their nymphal feeding, especially in areas such as southeastern Oregon where the prevalence of infection in cattle is thought to be high.

Quantitative PCR results suggest that once the gut of the tick becomes infected, there are no significant differences between populations in the number of *A. marginale* organisms in the guts of ticks. This is consistent with the observations of other authors: Eriks et al. (1993) reported that although there was variation between individual ticks in each group, once *A. marginale* colonized the gut, replication resulted in a mean level of organisms that was similar between groups, regardless of the infecting dose. This suggests that the factors that lead to differences in infection rate between populations, whether they have a genetic basis or not, may affect initial infection, but do not affect replication once the gut epithelial cells become infected. This implies that the variation between populations we have observed is related to initial events in the pathogen–epithelial cell interaction, such as a receptor-mediated interaction.

The number of ticks that developed salivary gland infections in this study was very low. Infection of the salivary glands is essential for transmission, however, it may be impossible to properly evaluate the salivary gland infection rate without a transmission feeding or a longer acquisition feeding to promote dissemination of *A. marginale* to the salivary gland. Previous findings suggest that either transmission feeding, or incubation at 32–37°C, stimulates higher levels of salivary gland infection and replication (Kocan et al. 1993). The ticks in the current study were acquisition fed for only 2 d on a persistently infected animal and then were incubated at 25°C for 8–10 d; although the guts became infected, the short acquisition feeding followed by incubation at room temperature did not stimulate high

levels of salivary gland infection. Presumably, a subsequent feeding would stimulate replication in the gut, followed by dissemination to and replication in the salivary glands, resulting in transmission. In a recent study, the salivary glands of ticks that were removed from their acutely infected acquisition host at intervals after attachment were only weakly positive by PCR for *A. marginale* starting at 2–5 d postattachment and were not strongly positive until day 6 (Lohr et al. 2002). Because that data were based on pools of 10 ticks, the weak positive result at day 2 suggests that salivary glands from one or a few of the ticks in the pool were positive at that time, whereas the strong positive at day 6 suggests that a larger proportion of the ticks have infected salivary glands after a longer feeding time. For ticks that acquire gut infection within the first 2 days of feeding, as in the current study, the remaining days of a longer acquisition feed may stimulate salivary gland infection in the same way that transmission feeding would.

Variation from one year to the next in the proportion of ticks that were susceptible to gut infection was not significant, and, in fact, at two of the sites (Miles City and Walker Lake) the proportion of susceptible ticks was the same from one season to the next, in spite of the 16-fold higher rickettsemia of the acquisition host in the second year. Although there were year-to-year differences at the two other sites (Lake Como and Placidea Butte), the differences were not significant and the trend was the same in both years, i.e., in both years Lake Como had the lowest susceptibility, Placidea Butte the highest, with Miles City and Walker Lake intermediate. Within populations, the size of the ticks clearly has no effect on infection. Even in the Como Lake population where the size variation was extreme (the largest tick was>5 times the size of the smallest tick), there was no significant difference between the weights of infected and uninfected ticks in both years of the study. The ticks from the Placidea Butte population were significantly smaller than those from all of the other three populations in both years of the study. The reasons for this significant size difference have not been investigated. Tick size has been shown to vary with altitude and season (Chaka et al. 1999). It also seems likely that adult size variation could be related to the diversity and the quality of the larval and nymphal hosts. Whatever the explanation for the smaller size of the Placidea Butte ticks, it seems unlikely that their size is related in any way to the greater rate of pathogen acquisition in this population, especially because larger and smaller ticks within each population are equally likely to become infected.

Whether there is a correlation between *D. andersoni* gut competence for *A. marginale* and the prevalence of infection in cattle in these regions is an intriguing question. Although prevalence data specific to the cattle herds that these tick populations are exposed to are not available, there is some historical data on the regional prevalence of *Anaplasma* infection. In a recent study, infection prevalence in the region of Montana that includes Miles City was 1.93%; in the part of Montana that contains Lake Como there was a 0.87% prevalence of infection (Van Donkersgoed et al. 2004). These were the two sites with the lowest level of tick susceptibility, 33.3 and 12.5%, respectively. In contrast, the prevalence of infection at Placidea Butte (formerly known as "Squaw Butte") was determined to be 71% in a study published in 1977 (Peterson et al. 1977), and at another Oregon site ≈200 km northeast of Placidea Butte, the prevalence was 64.5% in 1998 (Torioni de Echaide et al. 1998). In the current study, the tick susceptibility at Placidea Butte was the highest at 54.5–62.5%. Unfortunately, no prevalence data are available for Walker Lake where 50% of the ticks were susceptible. Cattle serosurveys have not been done in areas of Canada proximate to the Walker Lake site, although *A. marginale* is known to be present in Canadian bison (de la Fuente et al. 2003). It seems that infection prevalence in cattle may be correlated with tick susceptibility to initial midgut infection; however, without more data discussion of cause and effect would be speculative.

In conclusion, this study demonstrates that there is significant population level variation in *D. andersoni* midgut susceptibility to *A. marginale*, a key component of tick vector competence for this important tick-borne pathogen of cattle. This finding, in combination with studies examining the effect of *A. marginale* strain on transmissibility, may lead to an understanding of why both infection prevalence and disease incidence vary markedly within the region where *D. andersoni* is the predominant vector.

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